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Degradation of cocoa proteins into oligopeptides during spontaneous fermentation of cocoa beans

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ABSTRACT

Degradation products of proteins produced during fermentation are believed to be the key precursors of a range of Maillard reactions that deliver the characteristic flavor and aroma of cocoa and chocolate. We have utilized UPLC-ESI-Q-q-TOF to identify and relatively quantify the largest collection of cocoa oligopeptides during a spontaneous fermentation time series using Ivory Coast cocoa beans. Peptides were identified, sequenced by tandem mass spectrometry and annotated based on their characteristic fragmentation pattern in the positive-ion mode. This enabled us to quantitatively trace the sequential degradation of the two main cocoa storage proteins, namely, albumin and vicilin. We observed sequential proteolytic degradation forming longer peptides in the early stages of fermentation and an increasing number of shorter peptides at the latter stages of fermentation. Protein degradation is mediated by both *endo*- and exopeptidases degrading at either peptide termini. In excess of 800 fermentation peptides could be unambiguously identified, providing unprecedented mechanistic details of cocoa fermentation.

1. Introduction

The complex chemistry behind the characteristic color, taste, and aroma of cocoa and its related products has been under investigation for several decades. Cocoa flavors are known to be generated only upon the roasting of fermented cocoa beans (Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; Rohan, 1964; Voigt et al., 1994), which emphasizes the importance of the fermentation process for the final characteristics of the cocoa product (Biehl & Passern, 1982; Biehl, Wewetzer, & Passern, 1982). Several compounds responsible for the aroma (Frauendorfer & Schieberle, 2006, 2008; Ziegleder, 1991) and taste (Stark, Bareuther, & Hofmann, 2005, 2006) have already been identified and include for aroma, volatiles such as aldehydes, esters, pyrazines, as well as non-volatiles like diketopiperazines, proanthocyanidins, amino acids, organic acids, and sugars.

The key components of Maillard reactions first involve amino acids, short peptides, or more generally, molecules containing amine moieties, which react with aldehydic groups present in reducing sugars in a condensation and subsequent rearrangement reaction accompanied by the loss of a water molecule (Fayle & Gerrard, 2002). These initially formed so-called “Amadori” compounds can then undergo a cascade of reactions which ultimately yield the unique flavor, aroma, and color compounds that characterize a given food (Mottram & Taylor, 2011). In this regard, the proteogenic molecules participating in Maillard reactions are particularly responsible for the diversity of reaction products formed. It has been reported that the smaller oligopeptides could be much more reactive in Maillard reactions as compared to individual amino acids (de Kok & Rosing, 1993; Ho, Oh, Zhang, & Shu, 1992; Izzo & Ho, 1992). Furthermore, peptide hydrolysates have been shown to produce more flavor compounds than the corresponding amino acid lysates of the exact same protein mixtures (J. Liu, Liu, He, Song, & Chen, 2015; Liu et al., 2012). Specifically, in cocoa, these peptides are degradation products resulting from the fermentation-driven proteolytic cleavage of cocoa storage proteins, the most abundant of which are albumin and vicilin (representing ca. 90% of cocoa protein content) (Voigt, Biehl, & Wazir, 1993). Their degradation is triggered by the penetration of microbial fermentation by-products, such as ethanol, lactic acid, and particularly acetic acid, into the bean cotyledon, where proteolytic enzymes, namely, endopeptidase and carboxypeptidase, get activated along with unspecific protein cleavage reactions (Bytof, Biehl, Heinrichs, & Voigt, 1995; Voigt, Heinrichs, Voigt, & Biehl, 1994). While there have been several studies describing the breakdown of cocoa proteins, there have been only limited and very recent studies documenting the oligopeptides formed as a result of this degradation

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(Buyukpamukcu et al., 2001; Caligiani, Marseglia, Prandi, Palla, & Sforza, 2016; Janek, Niewienda, Wöstemeyer, & Voigt, 2016b; Marseglia et al., 2014; Mayorga-Gross, Quirós-Guerrero, Fourny, & Vaillant, 2016; Voigt, Janek, Textoris-Taube, Niewienda, & Wöstemeyer, 2016). Furthermore, these studies employed complicated extraction procedures, which most probably resulted in major losses of oligopeptides, and identified relatively few peptides (ca. 130 peptides), with a large number of molecules left unidentified or simply assumed to be peptides.

The formation of small peptides in cocoa fermentation offers other interesting aspects since small peptides have recently been discussed as compounds promoting health (Martorell et al., 2013; Paoletti, Poli, Conti, & Visioli, 2012; Raikos & Dassios, 2014). Numerous biological effects benefiting human health have been identified for small peptides originating from dairy processing or being formed by proteolytic degradation of proteins in the digestive tract. Cocoa represents a rich source of small peptides in our daily diet and a thorough characterization of its peptide profile offers many opportunities for future applications (Daliri, Oh, & Lee, 2017; Erdmann, Cheung, & Schröder, 2008; Korhonen & Pihlanto, 2003, 2006; Sánchez & Vázquez, 2017).

In this work, we have serendipitously identified a plethora of oligopeptides that are formed during the fermentation of cocoa and tracked their relative amounts at different fermentation stages in an effort to map the protein degradation in cocoa. Degradation was studied along a time series, which we refer to as fermentation series. In this fermentation series, samples were taken at the location of fermentation every day over a period of six days and all samples analyzed. This fermentation series allows a time-resolved mechanistic insight into the process of cocoa fermentation.

2. Material and methods

2.1. Chemicals and reagents

Dichloromethane, HPLC-grade acetonitrile, and HPLC-grade methanol were obtained from Carl Roth (Germany). Milli-Q water (18.2 MΩ·cm at 25 °C) was used throughout all experiments. Acetic acid (Product No. A6283) and Hesperetin (Product No. 51864) were obtained from Sigma-Aldrich.

2.2. Sample collection

Fresh cocoa pods from a single hybrid (G11UTA402XT413) were harvested on an estate (average tree age of 25 years) in the Ivory Coast (GPS 06° 48′ 37″N, 006° 59′ 10″). Pods were harvested, opened, placentas were separated, and spontaneous fermentation was initiated within 12 h from harvest. Two kilograms of fresh beans were isolated and frozen at −20 °C within 30 min of harvest. A hundred kilograms of these freshly harvested cocoa beans were box-fermented for a total of 168 h. Beans were turned at 48 h and 96 h after the start of fermentation. Around 500 g of beans were sampled at 24, 48, 72, 96, 120, 144, and 168 h respectively and were frozen at −20 °C. For convenience, these time points have been abbreviated throughout the manuscript as F1, F2, F3, F4, F5, F6, and F7, respectively. After fermentation, beans were sun-dried (ca. 7 days) and 2 kg of these dried fermented beans were also frozen at −20 °C. The dried beans had a moisture content of ca. 6% and a lipid content of ca. 56%. The entire fermentation time series was shipped under dry ice and stored at −80 °C before further processing. Defatted samples were stored at 4 °C prior to HPLC-MS analysis.

2.3. Sample preparation

Cocoa bean samples (at least 50 g of each) were de-shelled and ground to a fine powder using a knife mill (Retsch Grindomix GM200, Germany) at 10,000 rpm. Around 5 g of powder was defatted using dichloromethane in an automated Soxhlet extraction apparatus (Büchi B-811, Germany) for 18 h. The defatted powder was dried under vacuum and stored at 4 °C until further use.

Defatted powders were extracted using an acidified methanolic solution (MeOH:H_2O:CH_3COOH::70:28:2). An amount of 50 mg of defatted power was mixed with 5 mL of the extraction solution, ultrasonicated for 10 mins, and stirred for a further 30 mins at room temperature. This mixture was then syringe-filtered through a PTFE membrane filter (0.45 µm). The methanolic extract was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and used directly for HPLC-MS experiments.

2.4. HPLC-TOF-MS

HPLC experiments were performed on an Agilent 1260 HPLC system using a Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm particle size) along with the recommended guard column. The sample injection volume was 2 µL. The binary solvent system used consisted of Milli-Q water (Solvent A) and acetonitrile (Solvent B), both containing 0.05% formic acid. The gradient employed was adapted from a previously reported method (Sánchez-Rabaneda et al., 2003) at a constant flow rate of 0.5 mL/min and a column temperature of 30 °C. The gradient was (*t* (min), %B): (0, 8); (1, 8); (2.5, 12); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95).

The effluent HPLC system was connected to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an electrospray ionization source (nebulizer pressure of 1.8 bars, dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). All data were acquired in both negative- and positive-ion mode. Both full scan spectra and MS/MS datasets were recorded. The TOF analyzer was calibrated with a 0.1 M sodium formate solution before each chromatographic run. Monoisotopic molecular masses were calculated from molecular formulae using reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015).

Mass calibration and charge de-convolution of HPLC-TOF-MS data was performed using DataAnalysis 4.2 (Bruker, Germany) as well as the R package XCMS (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006; Tautenhahn, Bottcher, & Neumann, 2008) and MAIT (Fernández-Albert, Llorach, Andrés-Lacueva, & Perera, 2014). MGF (Mascot Generic Format) files were generated using DataAnalysis and mzML files were generated using the MSConvert tool bundled in the ProteoWizard Toolkit (Chambers et al., 2012). Peak areas from full scan spectra were normalized to the internal standard (hesperetin) and used for relative quantification.

2.5. Peptide identification

MGF files for each sample were used for peptide identification using a combination of several techniques. For longer oligopeptides, consisting of 8 or more amino acids, MGF files for all samples were loaded into the freely available software, SearchGUI (Vaudel, Barsnes, Berven, Sickmann, & Martens, 2011), which was used to initiate multiple searches on well-known peptidomics platforms, namely, MS-GF+ (Kim, Gupta, & Pevzner, 2008; Kim & Pevzner, 2014) and X!Tandem (Craig & Beavis, 2003, 2004; Fenyö & Beavis, 2003). The peptide search was initiated with the default parameters, except for the mass accuracy, which was conservatively altered to 5 ppm for precursor ions and 10 ppm for fragment ions. The protein sequences used for the search were obtained from the UniProt database, which was queried for all known proteins of *Theobroma cacao,* and resulted in a total of 40,949 sequences. The resulting data was analyzed using another freely available software, PeptideShaker (Vaudel et al., 2015), from which detailed parameters of the peptide search can be explored, including sequence coverage, matching fragments, as well several parameters for identification confidence. The complete fragmentation pattern of the